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Loss of filament-forming ability of myosin by non-enzymatic glycosylation and its molecular mechanism

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Abstract **Carp and scallop myosin and their subfragments (S-1 and rod) were reacted with glucose to investigate the effect of non-enzymatic glycosylation on the functionality of myosin. The filament-forming ability of the myosin rod diminished with the progress of non-enzymatic glycosylation and myosin became soluble in 0.1 M NaCl. The inhibition of the self-assembly of myosin molecules occurred chemically as a result of the increase in negative charge repulsion among myosin molecules and, further, physically as a result of the introduction of the glycosyl units into the surface of the rod region.**

Keywords: Myosin; Myosin rod; Filament formation; Non-enzymatic glycosylation; Reducing sugar

1. Introduction

Non-enzymatic glycosylation is a complex series of reactions between amino groups of proteins and reducing sugars that take place in living systems. The effect of non-enzymatic glycosylation on the structure and functionality of proteins has been studied in collagen [1], albumin [2], lens crystalline [3], and hemoglobin [4] in relation to aging and the complications of diabetes. Myosin, a highly conserved protein in eukaryotic cells that is especially abundant and ordered in striated muscles in animals, is also glycosylated in living systems [5]. Muscle contraction in higher eukaryotes is mediated by the sliding of interdigitating myosin and actin filaments. The myosin molecule consists of two subfragments, the globular head portion (water-soluble), called Subfragment-1 (S-1), and the long coiled-coil helical tail (salt-soluble), called rod [6]. S-1 has two functionally important sites, namely the actin binding site and the ATPase catalytic site. Since these sites are lysine-rich regions, the reaction with reducing sugars would affect the functionality of myosin. Indeed, Syrovy and Hodny [7,8] observed the loss of myosin ATPase activity with the progress of non-enzymatic glycosylation, and Ramamurthy et al. [9] observed the decrease in motility speed in muscle with the progress of non-enzymatic reaction between myosin and glucose. The rod region of myosin plays a significant role in the formation of the myosin filament [10]. Myosin molecules aggregate and assemble into insoluble filaments when they exist in low-ionic-strength media and neutral pH. Since muscle contraction occurs when actin filaments slide into the myosin filaments in myofibrils, the filament-forming ability of the myosin molecule has a quite important function in muscle contraction. However, there is little information about the effect of the glycosylation of the rod region on the filament formation of myosin in skeletal muscles.

Recently, our research group noted that fish and shellfish myosin in myofibrils became water-soluble in a physiological condition at the early stage of non-enzymatic glycosylation [11,12]. The objective of this study is to investigate the effect of non-enzymatic glycosylation on the filament formation of myosin. Carp and scallop myosin and their subfragments (S-1 and rod) were reacted with glucose and their solubility change and soluble state were examined to discuss the molecular mechanism of the loss of filament formation of myosin by non-enzymatic glycosylation.

2. Materials and methods

2.1. Materials

Live carp (*Cyprinus carpio*) and scallop (*Pecten yessoensis*) were obtained at a local fish market. Bovine serum albumin (fraction V) from Merck Co. Ltd. (Darmstadt, Germany), a calibration kit of glycosylated human serum from Japan Roche Diagnostic, Inc. (Tokyo, Japan), and Sephacryl S-400 from Pharmacia LKB Biotechnology (Uppsala, Sweden) were used in this study. All other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Preparation of myosin, S-1, and rod

Myosin was prepared from carp dorsal muscle and scallop striated adductor muscle [13]. The purified myosin was dissolved in 0.5 M NaCl and finally diluted slowly by adding 9 volumes of cold distilled water to prepare the myosin suspension in 50 mM NaCl (no buffer). Myosin S-1 and rod were prepared directly from the myofibrils by 1/250 (w/w) of α-chymotrypsin from bovine pancreas (EC 3.4.21.1 specific activity: 1000 U/mg) at 20 °C (carp) and 15 °C (scallop) for 30 min. The purified myosin S-1 and rod regions were suspended in 50 mM NaCl by dialysis at 4 °C for 16 h. The protein concentration was measured by the biuret method [14] with bovine serum albumin as a standard. Purity of proteins was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) [15].

2.3. Glycosylation of myosin, S-1, and rod

Reducing sugars (glucose, maltose, and maltotriose) at a final concentration of 0.3 M were mixed with the proteins (3.0 mg/ml) suspended in 50 mM NaCl, and immediately lyophilized using a freeze dryer. The lyophilized protein–sugar mixtures were incubated at 50 °C and 35% relative humidity using a temperature/humidity control cabinet to introduce the reducing sugars into the protein molecules via non-enzymatic glycosylation. In this study, a lyophilized myosin-sorbitol mixture was prepared and examined under the same condition as the control.

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2.4. Solubility of glycosylated proteins and their available lysine and fructosamine contents

Samples were dissolved in 0.1 and 0.5 M NaCl containing 20 mM Tris–HCl (pH 7.5) at 1.0–1.5 mg/ml of the final protein concentration with a homogenizer and immediately centrifuged at 15 000×*g* for 30 min at 4 °C. The protein solution before centrifugation and the supernatant were precipitated with 7.5% TCA. The precipitates were redissolved in 1.0 M NaOH and the protein concentrations were determined by the micro-biuret method [16] using bovine serum albumin as a standard. The solubility of the protein was expressed as a percent of the protein concentration in the supernatant with respect to that of the protein solution before centrifugation. Assays of available lysine [17] and fructosamine [18] were carried out to evaluate the progress of the non-enzymatic glycosylation between the protein and the reducing sugars. Before available lysine analysis, glycosylated proteins were precipitated with 7.5% TCA and redissolved in 2% SDS solution to remove unreacted sugars [11].

2.5. Structure of glycosylated proteins

Gel permeation chromatography was examined using Sephacryl S-400 column (16 × 90 cm) equilibrated by 0.1 or 0.5 M NaCl (pH 7.5) containing 0.05 mM phenylmethanesulfonyl fluoride. The α-helix content of the myosin rod was estimated by the following equation [19]: $\alpha\text{-helix}\ (\%) = (-[\theta]_{222\,nm} + 3000)/39\,000 \times 100$, where $[\theta]_{222\,nm}$ is the molecular ellipticity at 222 nm.

2.6. Determination of an isoelectric point

The isoelectric point (p*I*) of the myosin rod was estimated with DNA/protein analytical software (DNAsis, version 3.5, Hitachi Software Co. Ltd., Tokyo, Japan). The primary structure of the myosin rod was used to examine the p*I* of the proteins [20,21]. Urea–PAGE analysis [22] was carried out to confirm the change in net charge of the myosin rod by glycosylation.

2.7. Filament formation of the myosin rod

Change in the turbidity of the myosin rod by lowering the pH was measured to estimate the filament-forming ability of the myosin rod [23]. Samples were suspended in 0.1 M NaCl containing 20 mM Tris–maleate (pH 7.5) at 0.2 mg/ml of the final concentration and the turbidity at 350 nm was measured at 20 °C (for carp) and 15 °C (for scallop). Maleic acid at 0.25 M was used to decrease the pH of the myosin rod suspension.

2.8. Electron microscopy

The aliquot of the myosin rod suspension in 0.1 M NaCl containing 20 mM Tris–HCl (pH 7.5) was applied to a copper grid, negatively stained with 2% uranyl acetate, and observed with a Hitachi H-7000 electron microscope at an acceleration voltage of 75 kV.

3. Results and discussion

3.1. Solubility changes in myosin and its subfragments during reaction with glucose

Fish myosin was thermally less stable than that of other vertebrates and became insoluble with heat-denaturation. In this study, proteins were then reacted with glucose under lyophilized conditions to avoid heat-denaturation. The loss of available lysine and the production of fructosamine occurred simultaneously when the lyophilized proteins were incubated with glucose at 50 °C and 35% relative humidity (Fig. 1). There was no difference in the decreasing rate of the available lysine in the glycosylated subfragments in the

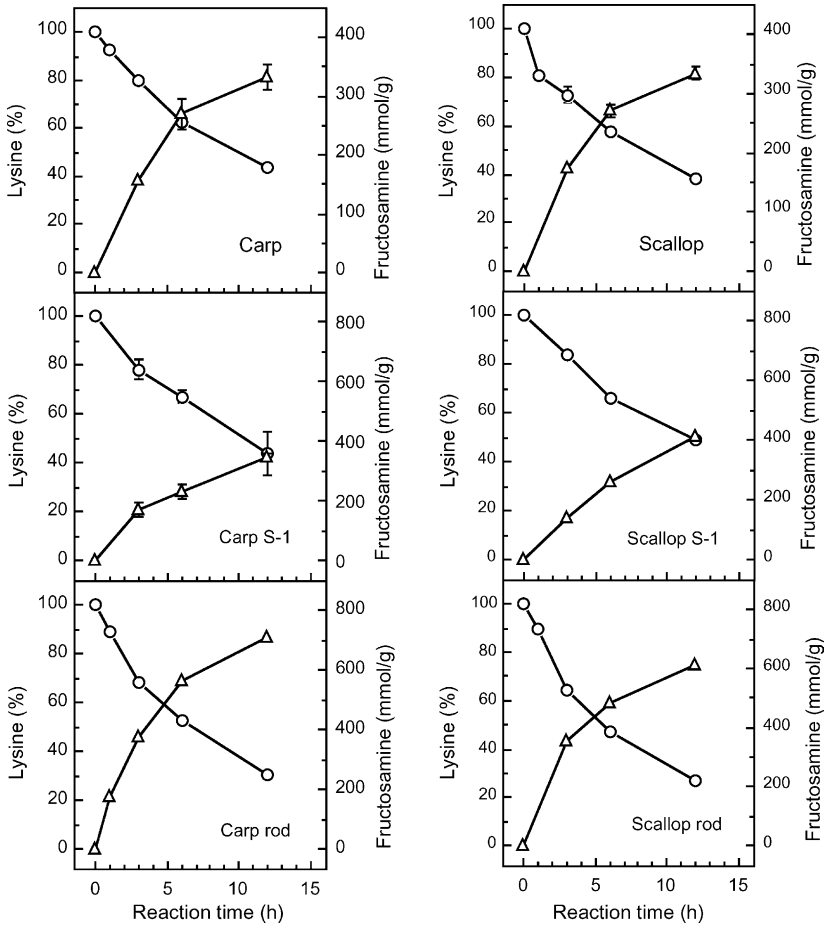

Fig. 1. Changes in available lysine and fructosamine contents of carp and scallop myosin subfragments during a reaction with glucose. Myosin, S-1, and rod were reacted with glucose at 50 °C for 0–12 h. Available lysine content (circle), fructosamine content (triangle).

same manner as for myosin. These results indicate that there is no difference between the reactivity of myosin and that of its subfragments.

The solubility of carp and scallop myosin in 0.1 M NaCl increased markedly with the reaction time (Fig. 2). The improved solubility became almost equal to the solubility in 0.5 M NaCl when about 60% of the available lysine was reacted with glucose. The solubility of S-1 in 0.1 M NaCl remained at a high level (>90%), regardless of the reaction with glucose. On the other hand, the solubility of the myosin rod in 0.1 M NaCl increased markedly and reached the same value as the solubility in 0.5 M NaCl. No protein degradation and no protein polymerization were observed in the SDS–PAGE analysis of the glycosylated myosin (data not shown), and it is known that the solubility characteristics of myosin are reflected in the ionic-strength dependence of the solubility of rod region. Therefore, the results of Fig. 2 indicate that the solubility improvement of myosin in a low-ionic-strength medium was caused by the reaction with glucose and that the water-solubilization of myosin reflects the functional change of the rod region.

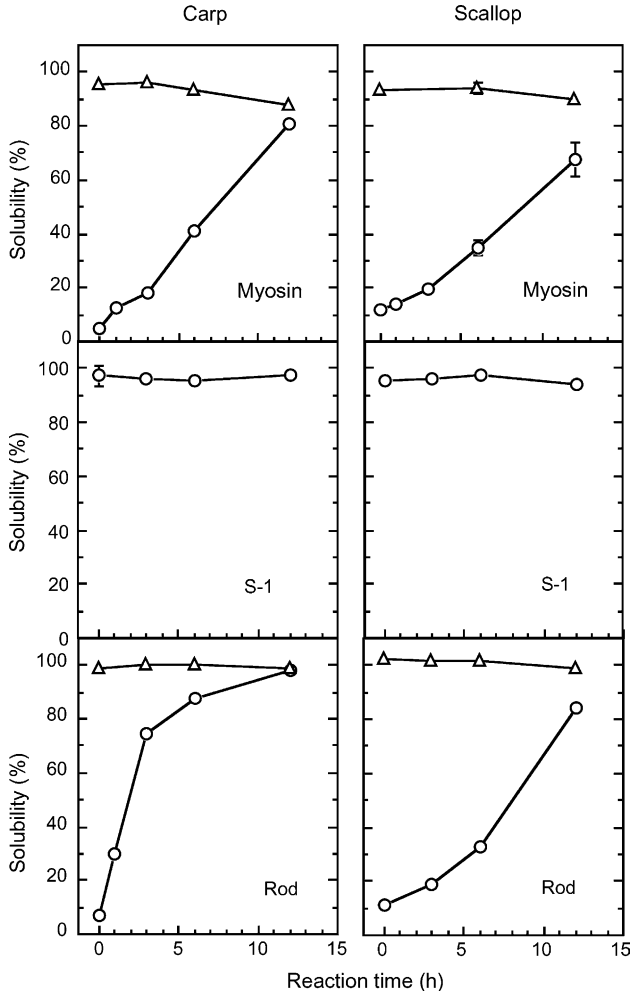

Fig. 2. Solubility changes in carp and scallop myosin and its subfragments during a reaction with glucose. Myosin, S-1, and rod reacted with glucose at 50 °C were dissolved in 0.1 M (circle) and 0.5 M (triangle) NaCl (pH 7.5).

3.2. Soluble state of the glycosylated myosin rod in a low-ionic-strength medium

In electron microscopy (Fig. 3), the native myosin rod of carp and scallop formed insoluble filaments in 0.1 M NaCl. However, the rod filaments disappeared completely when >70% of the lysine residues were reacted with glucose. Furthermore, in gel permeation chromatography analysis, the glycosylated myosin rods in 0.1 M NaCl were eluted as a single peak and coincided with the peak of native myosin rods dissolved in 0.5 M NaCl, which exist as a monomer (Fig. 4). These results indicate that the glycosylated myosin rods existed as a monomeric state in a low-ionic-strength medium.

3.3. Effect of pH on the filament-forming ability of the glycosylated myosin rod

The self-assembly of the myosin rod is accelerated by a decrease in negative charge repulsion [24]. The negative net charge of the myosin rod increases with the progress of the glycosylation because the positively charged lysine residues (ε-amino groups) are lost as a result of the reaction with glucose. It is estimated that the p*I* of the myosin rod was shifted from 4.98 to 4.17 (carp) and from 5.03 to 4.29 (scallop) when 69% and 73% of the available lysine was reacted with glucose and the improved solubility was 97% and 83%, respectively (Figs. 1 and 2). Indeed, the increase in the negative net charge of the myosin rod was confirmed by urea–PAGE analysis (data not shown). Therefore, it is possible that the loss of the filament formation of glycosylated myosin was related to an increase in the negative charge repulsion among myosin rod regions.

The pH of the myosin rod solutions (pH 7.5) shifted to the acidic side by the addition of maleic acid, and the turbidity changes of the solutions were measured to discuss the relationship between the water-solubilization and the increase in the negative net charge by the glycosylation (Fig. 5). The turbidity of the native myosin rod increased markedly by the

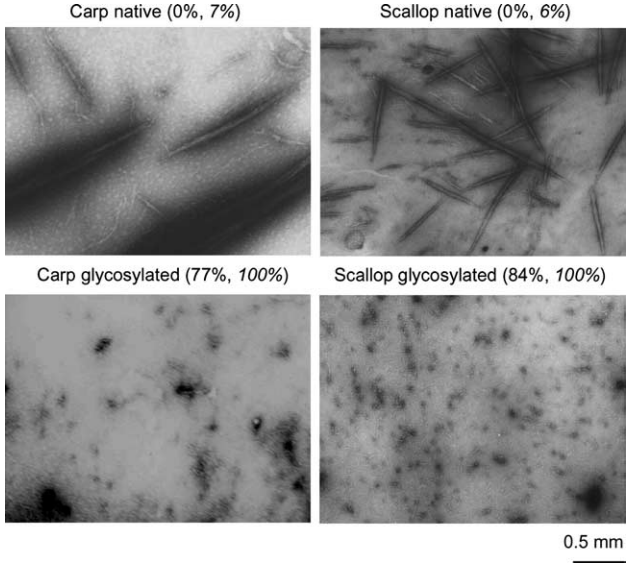

Fig. 3. Electron micrographs of carp and scallop myosin rods reacted with glucose. Myosin rod reacted with glucose at 50 °C for 24 h was dissolved in 0.1 M NaCl (pH 7.5). Numbers in parentheses are the rate of the available lysine reacted with glucose and the solubility in 0.1 M NaCl (*italics*).

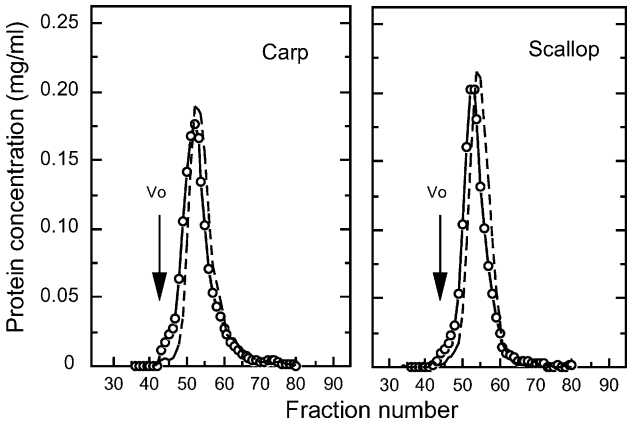

Fig. 4. Elution patterns of carp and scallop myosin rods reacted with glucose. Myosin rod reacted with glucose at 50 °C for 24 h was dissolved in 0.1 M NaCl (pH 7.5). and native rod (dotted line) dissolved in 0.5 M NaCl (pH 7.5) was monitored. V_o: void volume determined by actomyosin. Flow rate: 0.4 ml/min.

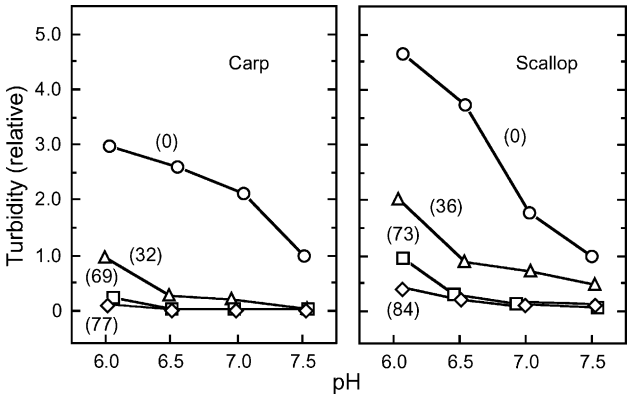

Fig. 5. Effect of pH on the filament formation of carp and scallop myosin rods reacted with glucose. Native rod (circle) and rod reacted with glucose for 3 h (triangle), 12 h (square), and 24 h (diamond) were allowed to form filament at 0.1 M NaCl (pH 7.5) by lowering the pH from 7.5 to 6.0. The numbers in parentheses are lysine residues reacted with glucose (%).

lowering of pH because the decrease in negative charge accelerated the filament formation of the myosin rod. Increase in turbidity at low pH conditions was also observed in the glycosylated myosin rods when about 30% of the available lysine was reacted with glucose. That is, it seems that the loss of filament-forming ability at the initial stage of glycosylation was caused by an increase in negative net charge repulsion. However, the degree of the turbidity increase diminished clearly with the progress of the glycosylation, and the lowering of the pH had no effect on the turbidity when >70% of the available lysine was reacted with glucose. Therefore, glycosyl units conjugated with proteins would also affect the water-solubilization of the myosin rod.

3.4. *Effect of glycosyl unit size on the solubility improvement of the myosin rod*

Table 1 shows the effect of the reaction with reducing sugars on the structure of the myosin rod. No change was observed in the α-helix content of the glycosylated myosin rod, whereas the filament-forming ability was lost, as pointed out in Figs. 3–5. The coiled coil structure also remained unchanged because no significant change was observed in the CD-spectra of the myosin rod during glycosylation and $[\theta]_{222\,nm}/[\theta]_{208\,nm}$ ratio was always >1.0 [25]. Therefore, it is apparent that the dissociation of myosin rod filaments was achieved without substantial conformational change of the rod region. The structure of the myosin rod is a two-strand α-helical coiled coil and the lysine residues in the myosin rod are located on the periphery of the coiled coil. This information suggests that glycosyl units located on the surface of the rod molecules could inhibit the self-assembly of myosin rod molecules. To confirm the hypothesis, maltose and maltotriose were reacted with the myosin rod in the same manner as for glucose, and the change in filament-forming ability during glycosylation was examined to investigate the relationship between the solubility improvement and glycosyl unit size introduced into the rod region. All myosin rods reacted with the reducing sugars were highly solubilized in 0.1 M NaCl (Fig. 6), whereas the loss of available lysine and the production of the fructosamine occurred slowly as the molecular size of the glycosyl unit increased (data not shown). However, as shown in Fig. 6, the water-solubilization of the myosin rod was effectively achieved at lower decreasing rate of the lysine residues, with an increase in the glycosyl unit size. In the case of carp myosin rod, high solubility (>80%) was obtained when 10%, 15%, and 45% of the available lysine was reacted with maltotriose, maltose, and glucose, respectively. Additionally, the same trend was also observed in scallop myosin rod. From the data of Table 1, it is apparent that the introduced glycosyl units were located on the surface of the myosin rod molecules. Therefore, the result of Fig. 6 indicates that glycosyl units with larger molecular size interfere with interaction among myosin rods effectively. The glycosyl units introduced to the myosin rod region would act as a physical barrier and inhibit the filament formation of the rod region. Since the functionality (filament-forming ability and actin binding capacity) of carp and shellfish muscle proteins is basically the same as that of the mammalian species, the present study provides useful information on the pathophysiological changes of muscle function associated with non-enzymatic glycosylation.

In conclusion, our study confirmed that the non-enzymatic glycosylation, even at the early stage, impairs the filament formation of myosin. The inhibition of the self-assembly of myosin molecules occurred chemically by the increase in negative charge repulsion among myosin molecules and, further,

Table 1
α-Helix content of glycosylated myosin rod

Specimen	Glycosyl unit	Reaction time (h)[a]	Reacted lysine (%)	α-Helix (%)
Carp	–	0	0	90.4
	Glucose	24	77.4	85.6
	Maltose	24	33.3	92.1
	Maltotriose	48	18.4	90.6
Scallop	–	0	0	87.1
	Glucose	24	84.4	85.1
	Maltose	24	33.3	81.0
	Maltotriose	48	18.5	87.3

[a] Reaction was performed at 50 °C and 35% relative humidity.

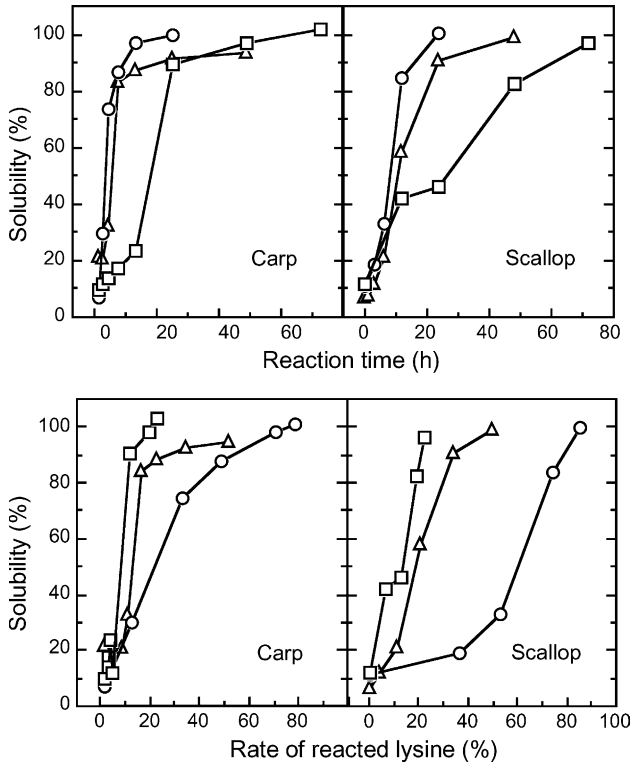

Fig. 6. Effect of the glycosyl unit size on solubility changes of carp and scallop myosin rods in 0.1 M NaCl (pH 7.5) during reactions with glucose, maltose, and maltotriose. Myosin rods were reacted with glucose (circle), maltose (triangle), and maltotriose (square) at 50 °C for 0–72.

physically, by introducing the glycosyl units into the surface of the rod region.

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